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Expression of Equi Merozoite Antigen 2 during Development of *Babesia equi* in the Midgut and Salivary Gland of the Vector Tick *Boophilus microplus*

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Equi merozoite antigens 1 and 2 (EMA-1 and EMA-2) are *Babesia equi* proteins expressed on the parasite surface during infection in horses and are orthologues of proteins in *Theileria* spp., which are also tick-transmitted protozoal pathogens. We determined in this study whether EMA-1 and EMA-2 were expressed within the vector tick *Boophilus microplus*. *B. equi* transitions through multiple, morphologically distinct stages, including sexual stages, and these transitions culminate in the formation of infectious sporozoites in the tick salivary gland. EMA-2-positive *B. equi* stages in the midgut lumen and midgut epithelial cells of *Boophilus microplus* nymphs were identified by reactivity with monoclonal antibody 36/253.21. This monoclonal antibody also recognized *B. equi* in salivary glands of adult *Boophilus microplus*. In addition, quantification of *B. equi* in the mammalian host and vector tick indicated that the duration of tick feeding and parasitemia levels affected the percentage of nymphs that contained morphologically distinct *B. equi* organisms in the midgut. In contrast, there was no conclusive evidence that *B. equi* EMA-1 was expressed in either the *Boophilus microplus* midgut or salivary gland when monoclonal antibody 36/18.57 was used. The expression of *B. equi* EMA-2 in *Boophilus microplus* provides a marker for detecting the various development stages and facilitates the identification of novel stage-specific *Babesia* proteins for testing transmission-blocking immunity.

Pathogens in the genus *Babesia* cause acute disease in animals and humans (7, 9, 11). Unlike the closely related *Plasmodium* spp. that cause malaria and are transmitted by mosquitoes, *Babesia* spp. develop within ixodid ticks (1, 27). The development of *Babesia* spp. within the tick begins in the midgut lumen and culminates in the formation of infectious sporozoite stages within the salivary gland acini (8, 18, 25). This development is illustrated by *Babesia equi*, a pathogen of horses (20, 30). Following ingestion of *B. equi* merozoites into the tick midgut, the parasites are stimulated to undergo gametogenesis and are fertilized to form zygotes (23, 24, 30). The zygote then adheres to and invades midgut epithelial cells and transforms into a kinete (22). Mature kinetes egress to the hemocoel and invade salivary gland acinar cells, where they undergo sequential transformations into sporonts, intermediate-form sporoblasts, and ultimately mature sporozoites, the infective stage for the mammalian host (7, 8, 9, 25). This complex development requires not only transition from a vertebrate to an invertebrate environment but also invasion of multiple distinct cell types, including erythrocytes, tick midgut epithelial cells, and tick salivary gland epithelial cells. Mediating these events is postulated to involve expression of common surface proteins throughout multiple distinct stages of the parasite or regulation of novel surface molecules by the distinct life cycle stages within the mammalian host and the vector.

In contrast to stage-specific surface molecules needed to mediate events such as attachment and invasion in the mammalian versus invertebrate host (5, 6, 16, 28), surface proteins expressed in multiple stages may mediate a function in both the mammalian host and the vector tick. Erythrocyte stages of *B. equi* express immunodominant surface proteins designated equi merozoite antigens (2, 13, 15). Equi merozoite antigen 1 (EMA-1; 34 kDa) and EMA-2 (30 kDa) are each encoded by paralogous single-copy genes and are expressed by intraerythrocytic parasite stages (10, 13). The high degree of similarity between EMA-1 and EMA-2 is consistent with the two proteins having a common function or closely related functions within the mammalian host. To test whether these merozoite surface molecules are expressed at critical stages within the vector tick, we examined EMA-1 and EMA-2 expression in the midgut of *Boophilus microplus* nymphs following acquisition feeding, and then in the salivary gland of *Boophilus microplus* adults at the time when infective sporozoites develop.

To maximize the levels of *B. equi* within *Boophilus microplus*, nymphal feeding was synchronized with ascending *B. equi* parasitemia. A splenectomized pony (H069) was inoculated with 2 ml of the Florida strain of *B. equi* (approximately 8×10^7 infected erythrocytes), a strain that has been shown to be transmissible by *Boophilus microplus* (14). To determine the number of *B. equi* parasites during early ascending parasitemia, real-time PCR was performed with primers derived from the single-copy gene *ema-1* of *B. equi* (4, 13). It was predicted that the *ema-1* primers (forward, 5′-GAGTCCATT GACCACGTCACC-3′; reverse, 5′-GTGCCTGACGACAGT CTTTGG-3′) would amplify a fragment from nucleotide 115

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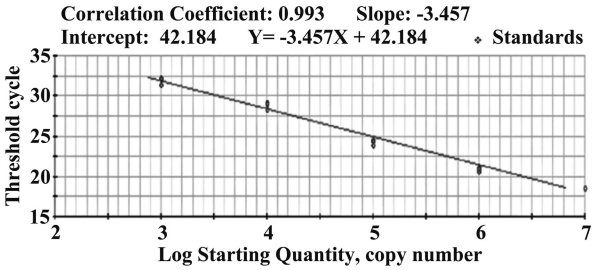

FIG. 1. Real-time PCR detection of the *B. equi ema-1* gene. The standard curve was generated by using a pBluescript SK(−) vector with the full-length *ema-1* gene (14). The *x* axis indicates the log starting quantity of the template. The efficiency of amplification was 0.95.

to 270. The fluorogenic probe 5′-TCGACAAGCAGTCCGA GGAGCACA-3′ (PE Applied Biosystems, Foster City, Calif.) was generated to anneal between bases 137 and 161 of *ema-1*. The TaqMan assay was carried out under the following conditions: 95°C for 10 min, 50 cycles of 95°C for 20 s and 62°C for 15 s, final extension at 72°C for 7 min, and holding at 10°C. The reactions were conducted with a PCR mixture containing 10 mM Tris (pH 8.3); 50 mM KCl; 2.0 mM $MgCl_2$; 200 μM dATP, dCTP, dGTP, dTTP; a 0.2 μM concentration of each primer; 0.12 μM fluorogenic probe; and 1.25 U of AmpliTaq Gold (PE Applied Biosystems). All reactions were performed with the iCycler iQ real-time PCR detection system (Bio-Rad Laboratories, Hercules, Calif.). A standard curve of 10^3 to 10^7 *ema-1* copies (Fig. 1) was generated by amplifying the previously reported recombinant *ema-1* plasmid (14, 17). The efficiency of amplification using serial dilutions of the recombinant *ema-1* plasmid ranged from 0.93 to 0.99 (12). Test samples from H069 were amplified simultaneously with the set of standards to determine the level of parasites in the peripheral blood. Real-time PCR was performed with genomic DNA isolated from 100 μl of blood by a commercially available method (Gentra Systems, Inc., Minneapolis, Minn.). The levels of *B. equi* in the peripheral blood, analyzed in triplicate, are presented as the mean logarithms of the numbers of *B. equi* parasites per microliter of blood. There were $10^{0.96}$ *B. equi* parasites per μl of blood in pony H069 on day 6 postinoculation; this number rose to $10^{6.2}$ per μl by day 15 postinoculation. In contrast to the

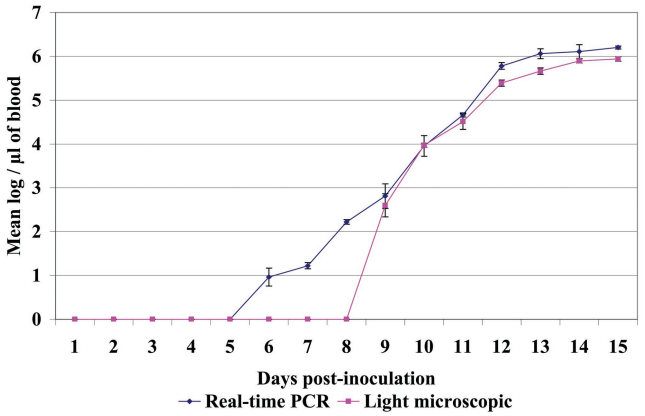

FIG. 2. Real-time PCR detection of early ascending *B. equi* parasitemia. The means and standard deviations of triplicate assays for both real-time PCR and light microscopic quantification of the parasites are indicated. *Boophilus microplus* nymphs were applied from days 3 through 7 postinoculation and were harvested from days 9 through 15 postinoculation.

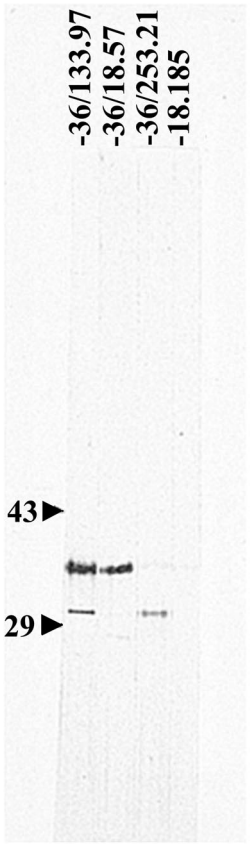

FIG. 3. Specificity of MAbs 36/133.97, 36/18.57, and 36/253.21. Immunoaffinity-purified native EMA-1 and EMA-2 from *B. equi* erythrocyte stages were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and incubated with individual MAbs. The immunoblotted proteins were probed with MAb 36/133.97 against an epitope common to EMA-1 and EMA-2, MAb 36/18.57 against EMA-1, MAb 36/253.21 against EMA-2, and the isotype-matched control MAb 18.185 against *C. parvum*. The 43- and 29-kDa molecular size standards are indicated on the left.

TABLE 1. Association between *B. equi* parasitemia level and presence of EMA-2-positive *B. equi* in the midgut

dpi[a]	Mean log parasites/ μl of blood	% of nymphs that contained EMA-2-positive *B. equi* in the midgut (no. positive/no. examined; dpa[b])
9	2.81	23.5 (4/17; 6)
10	3.95	30.4 (7/23; 6)
11	4.66	24.5 (14/57; 6)
12	5.78	60.0 (15/25; 6)
13	6.06	65.6 (44/67; 7)
14	6.11	83.3 (20/24; 7)
15	6.20	100 (10/10; 8)

[a] dpi, days postinoculation.
[b] dpa, days postattachment.

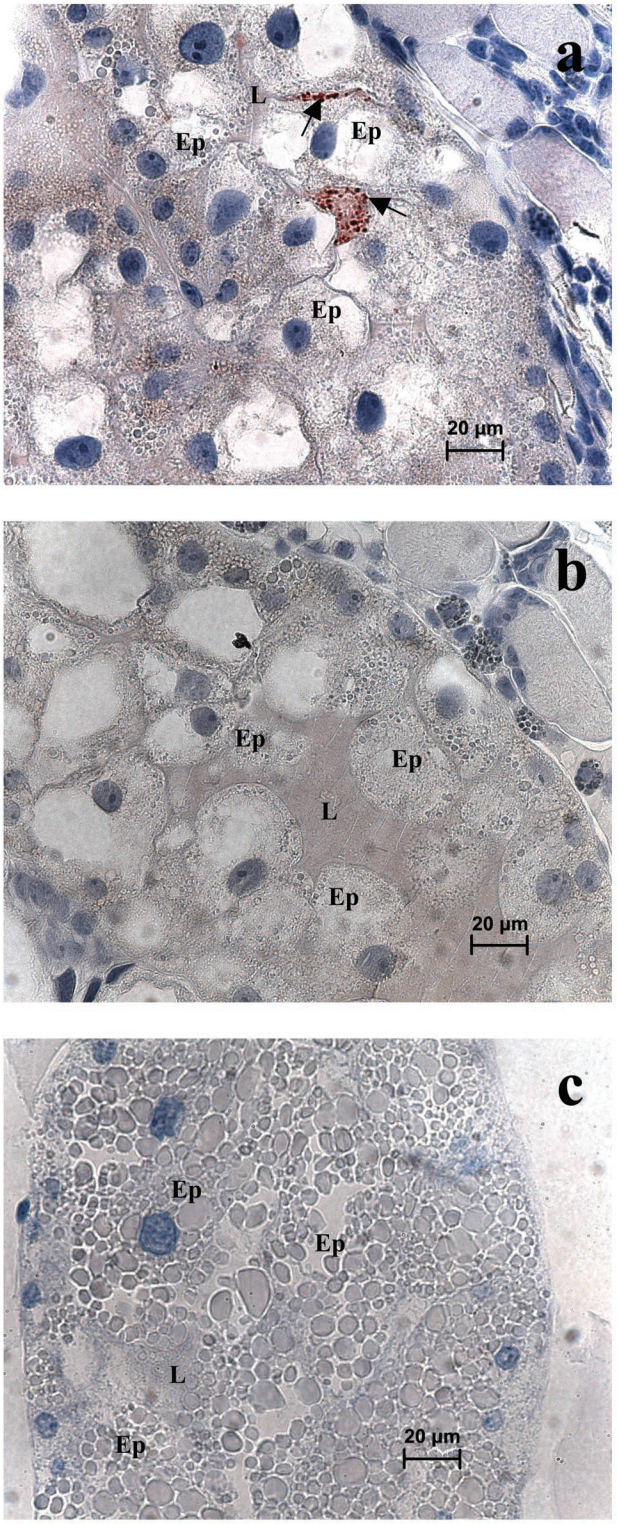

FIG. 4. Expression of EMA-2 by *B. equi* within the midgut lumen of *Boophilus microplus* nymphs fed during parasitemia levels below $10^{5.7}$ per μl of blood. (a) Semithin section of an infected nymph probed with MAb 36/253.21; (b) sequential section of an infected nymph probed with isotype-matched control MAb 18.185; (c) section of an uninfected nymph probed with MAb 36/253.21. L, midgut lumen; Ep, midgut epithelial cells. Arrows indicate *B. equi* in the midgut lumen.

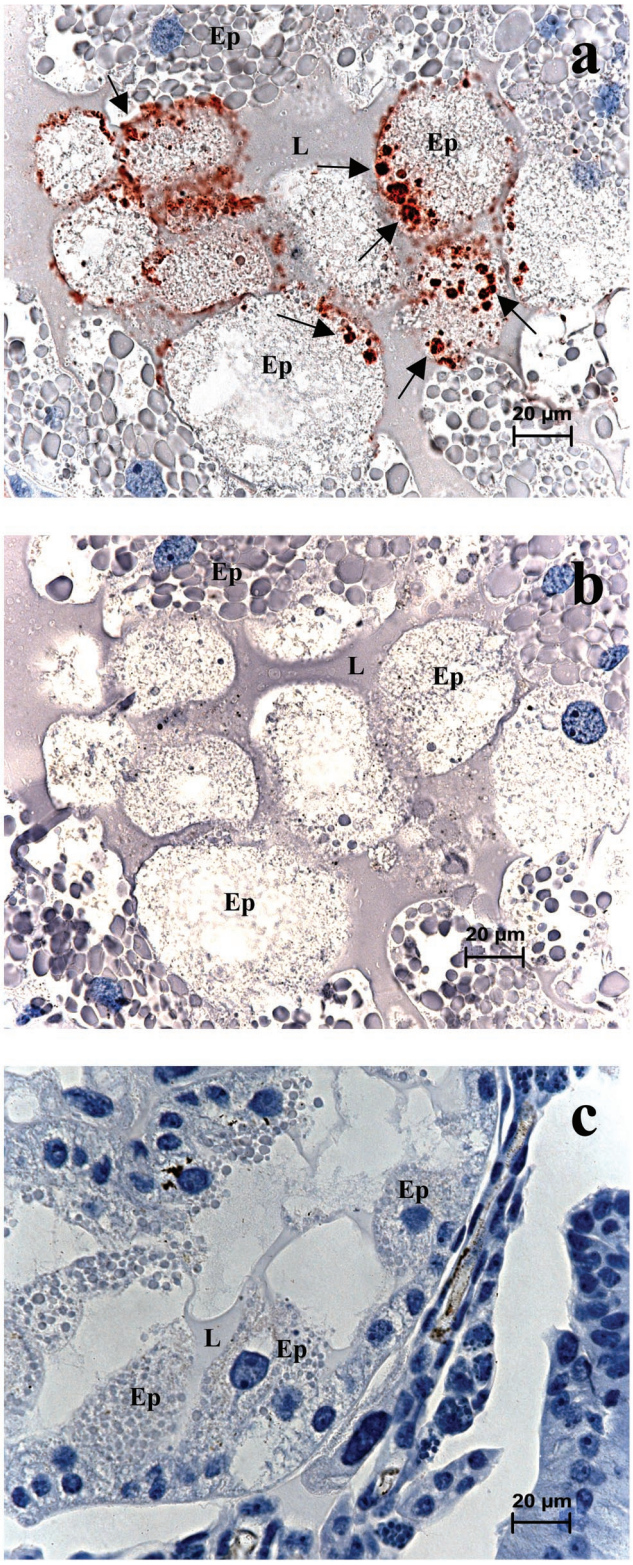

FIG. 5. Expression of EMA-2 by *B. equi* within the midguts of *Boophilus microplus* nymphs fed during parasitemia at levels exceeding $10^{5.7}$ parasites per μl of blood. (a) Semithin section of an infected nymph probed with MAb 36/253.21; (b) sequential section of an infected nymph probed with isotype-matched control MAb 18.185; (c) section of an uninfected nymph probed with MAb 36/253.21. L, midgut lumen; Ep, midgut epithelial cells. Arrows indicate *B. equi* in the midgut.

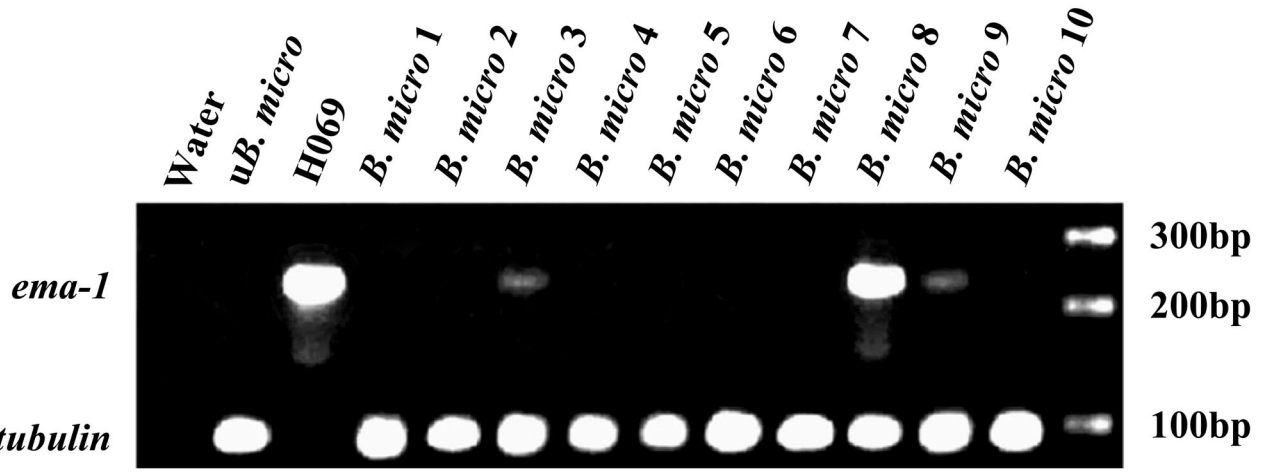

FIG. 6. Identification of *B. equi*-infected salivary glands from individual *Boophilus microplus* adults exposed as nymphs. u*B. micro*, salivary glands uninfected *Boophilus microplus*; H069, infected pony; *B. micro* 1 to 10, salivary glands from individual *Boophilus microplus* ticks. Molecular size markers are indicated at the right.

initial detection by real-time PCR on day 6, *B. equi* in the peripheral blood was first detected by light-microscopic examination of Giemsa-stained smears on day 9 postinoculation (Fig. 2). During the rise in parasitemia from days 9 through 15 postinoculation, there was close agreement between the number of parasites detected by real-time PCR and the number detected by microscopic examination (Fig. 2).

To generate *Boophilus microplus* nymphs, larvae were allowed to feed on a normal calf for 5 days. Engorged larvae were harvested and induced to molt to the nymphal stage within 48 h at 26°C, with 93% relative humidity and a 12-h photoperiod. Unfed nymphs were applied under individual cloth patches on H069 on days 3, 4, 5, 6, and 7 postinoculation and allowed to feed for a minimum of 2 days and a maximum of 8 days during ascending *B. equi* parasitemia. Nymphs were harvested at days 9 through 15 after *B. equi* inoculation during parasitemia levels between $10^{2.8}$ to $10^{6.2}$ per μl of blood (Fig. 2). Negative control *Boophilus microplus* nymphs were obtained by using the same colony and fed identically on an uninfected pony.

Following nymphal acquisition feeding, we tested whether EMA-1 and EMA-2 were expressed during the development of *B. equi* within *Boophilus microplus* nymphs. Partially fed nymphs harvested on days 9 through 15 postinoculation were promptly fixed in 10% formaldehyde for 8 to 18 h and embedded in paraffin. Following deparaffinization in Clear-Rite (Richard-Allan Scientific, Kalamazoo, Mich.), 4-μm sections were treated with target retrieval solution (DAKO Corp., Carpinteria, Calif.) and steam for 20 min and then cooled at room temperature prior to immunostaining (19, 21, 26). The tick sections were blocked with 5% normal goat serum in 0.01 M phosphate-buffered saline containing NaN_3. *B. equi*-specific monoclonal antibodies (MAbs) 36/18.57 (5 μg/ml) against EMA-1, 36/253.21 (0.1 μg/ml) against EMA-2, and 36/133.97 (5 μg/ml) against EMA-1 and EMA-2 (Fig. 3) were applied to tick sections, and the sections were incubated at room temperature for 25 min. The hybridomas secreting these MAbs were generated from BALB/c mice immunized with viable *B. equi* erythrocyte stages as previously described (15). Goat anti-mouse immunoglobulin G (Signet Pathology Systems, Inc., Dedham, Mass.) was applied following the primary antibodies, and the tick sections were incubated at room temperature for 25 min. The tick sections were treated with 3% hydrogen peroxide in distilled water and incubated at room temperature for 6 min. Following the hydrogen peroxide treatment, streptavidin-horseradish peroxidase was applied to the sections, and the sections were incubated at room temperature for 25 min. The substrate was 3-amino-9-ethylcarbazole containing hydrogen peroxide (DAKO Corp.), and tick sections were incubated at room temperature for 7 min. All sections were counterstained with Mayer's hematoxylin and mounted on coverslips with an aqueous mounting medium.

B. equi-specific MAbs 36/253.21 (anti-EMA-2) and 36/133.97 (anti-EMA-1 and -2) recognized *B. equi* forms 1.5 to 5 μm in diameter in the midgut of exposed nymphs. No reactivity was observed in sections of uninfected nymphs probed with either antibody or in sections of infected nymphs probed with isotype-matched control MAb 18.185 against *Cryptosporidium parvum*. The anti-EMA-1 MAb 36/18.57 showed no reactivity in sections of infected nymphs or in the *B. equi* erythrocyte stages concentrated in 1% agarose, fixed in 10% formaldehyde, and embedded in paraffin. The percentage of fed nymphs that contained EMA-2-positive *B. equi* was positively associated with the parasitemia level (Table 1). In addition to the percentage of nymphs containing *B. equi*, the morphology and location of the EMA-2-positive organisms within the midgut also varied in association with the parasitemia level and duration of feeding. Nymphs fed on pony H069 with parasite levels in the peripheral blood below $10^{5.7}$ per μl of blood contained *B. equi* forms 1.5 to 2.5 μm in diameter in the midgut lumen only (Fig. 4). These forms were morphologically indistinguishable from the *B. equi* erythrocyte stages. In contrast, nymphs fed when parasitemia exceeded $10^{5.7}$ per μl of blood contained *B. equi* forms of up to 5 μm in diameter in both the midgut lumen and midgut epithelial cells (Fig. 5), indicating the development of morphologically distinct stages. In addition to the

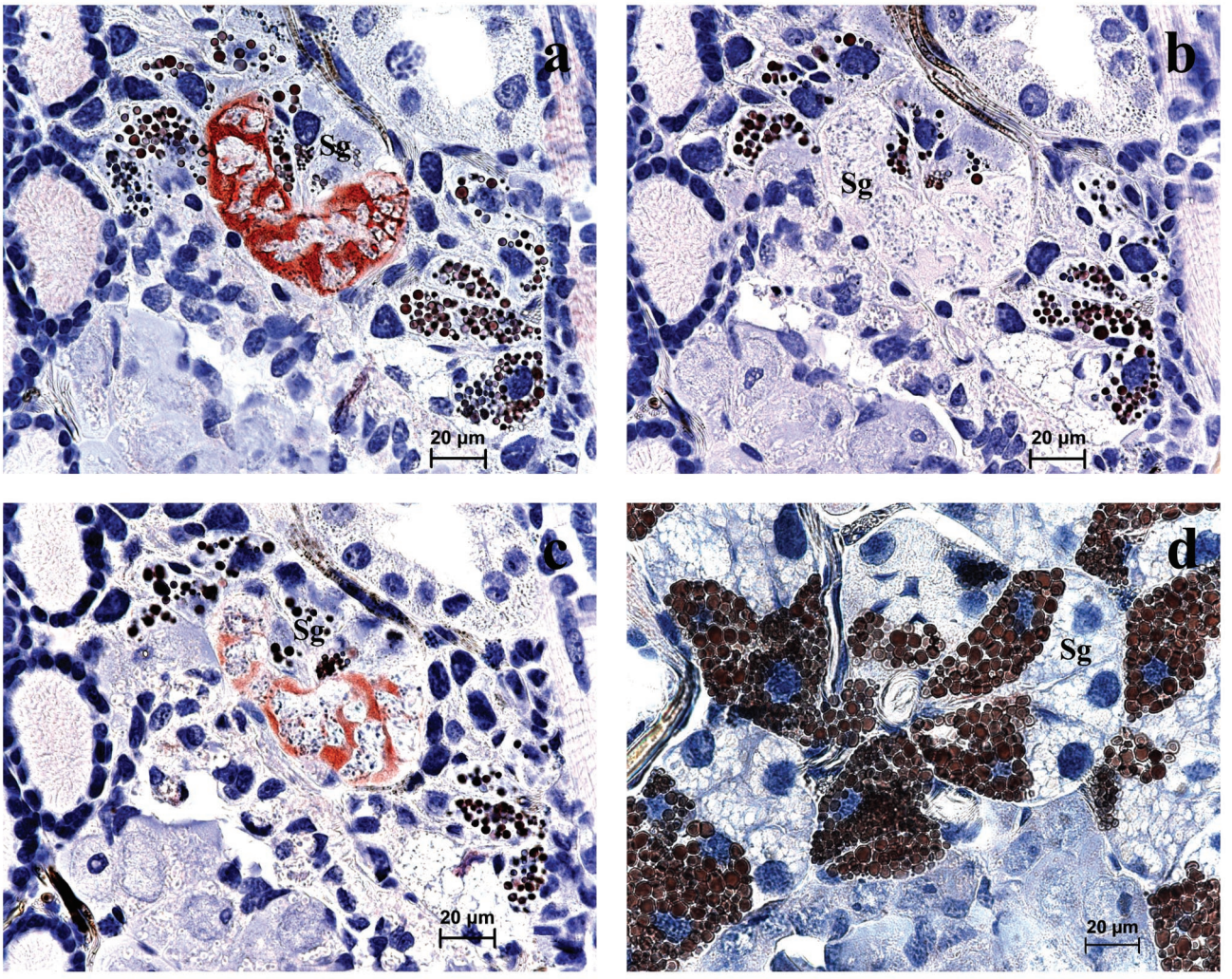

FIG. 7. Expression of EMA-2 by *B. equi* within the salivary glands of *Boophilus microplus* adults. (a) Semithin section of an infected *Boophilus microplus* adult probed with MAb 36/253.21; (b) sequential semithin section of an infected *Boophilus microplus* adult probed with isotype-matched control MAb 18.185; (c) sequential semithin section of an infected *Boophilus microplus* adult probed with MAb 36/133.97; (d) semithin section of an uninfected *Boophilus microplus* adult probed with MAb 36/253.21. Sg, salivary gland.

influence of parasitemia on *B. equi* stages in the midgut, the duration of tick feeding also appeared to affect the development of *B. equi* in the midgut. Nymphs fed for 2 to 3 days at parasitemia levels below $10^{3.9}$ per μl of blood showed no reactivity of antibodies in the midgut. The percentage of nymphs which had detectable *B. equi* within the midgut was 26.4% when nymphs were fed for 4 to 6 days at parasite levels below $10^{5.7}$ per μl of blood. In contrast, 60% of nymphs fed for 6 days at parasite levels exceeding $10^{5.7}$ per μl of blood contained *B. equi*. The highest percentage of nymphs containing *B. equi* in the midgut (66 to 100%) was observed when nymphs were allowed to feed for 7 or 8 days at parasitemia levels exceeding $10^{5.7}$ per μl of blood (Table 1).

Having identified the expression of EMA-2 by *B. equi* within the midgut of *Boophilus microplus* nymphs, we examined whether EMA-1 and EMA-2 were expressed by *B. equi* during development in the salivary glands of *B. microplus* adults. The engorged nymphs, infected by feeding on H069 for 9 to 11 days during ascending parasitemia, were harvested and induced to molt to the adult stage within 48 to 72 h by incubation at 26°C, 93% relative humidity, and a 12-h photoperiod. The adults were allowed to feed on a normal calf for 48 h to stimulate the development of *B. equi* in the salivary gland acinar cells (29). To determine if *Boophilus microplus* ticks exposed to *B. equi* as nymphs were infected, adult ticks were dissected and the salivary glands were isolated. Duplex nested PCR was carried out with genomic DNA from 10 individual salivary gland pairs extracted by a commercially available method (Gentra Systems, Inc.). The PCR mixtures contained two sets of primers, one to amplify the *Boophilus microplus* α-*tubulin* gene and the other to amplify *B. equi ema-1* (3, 10). It was predicted that *Boophilus microplus* α-*tubulin* primers (forward, 5′-CGTGCC GTATTTGTTGATC-3′; reverse, 5′-AGATTAGCTGCTCCG GGTG-3′) would amplify a fragment of 91 bp. It was predicted that the *ema-1* external primers (forward, 5′-CCGAGGAGG AGAAACCCA-3′; reverse, 5′-CGCCATAGACGGAGAAG

CC-3′) would amplify a fragment from nucleotide 165 to 778. The PCR was carried out under the following conditions: 94°C for 5 min; 30 cycles of 95°C for 30 s, 62°C for 30 s, and 72°C for 20 s; final extension at 72°C for 7 min; and holding at 4°C. The reaction was conducted in 25 μl containing 5 μl of extracted genomic DNA, a 1.6 μM concentration of each primer set, and 12.5 μl of PCR Master (Roche Diagnostics Corp., Nutley, N.J.). It was predicted that the *ema-1* internal primers (forward, 5′-AAGGACAACAAGCCATACGT-3′; reverse, 5′-TTGCCTGGAGCCTTGAAG-3′) would amplify a fragment of 226 bp (nucleotides 274 to 500). The duplex nested PCR was carried out under the following conditions: 94°C for 5 min; 25 cycles of 94°C for 15 s, 62°C for 20 s, and 72°C for 15 s; final extension at 72°C for 7 min; and holding at 4°C. The reaction was conducted in 25 μl containing 1 μl from the first reaction, a 2 μM concentration of each primer set (*ema-1* internal primers and *Boophilus microplus* α-*tubulin* primers), and 12.5 μl of PCR Master. The duplex nested PCR products were analyzed following 2% agarose gel electrophoresis. All salivary glands from adult ticks revealed the expected band for α-*tubulin* amplification and 3 of 10 contained *B. equi* amplicons (Fig. 6).

Having confirmed the infection of adult ticks by PCR, we examined whether EMA-1 and EMA-2 were expressed by *B. equi* during development in the salivary glands. Adult ticks that had been fed to stimulate *B. equi* development in the salivary gland were fixed in 10% formaldehyde and embedded in paraffin. Semithin sections of *Boophilus microplus* adults exposed to *B. equi* as nymphs were probed with antibodies 36/18.57, 36/253.21, and 36/133.97. *B. equi*-specific MAbs 36/253.21 (anti-EMA-2) and 36/133.97 (anti-EMA-1 and -2) recognized *B. equi* in the salivary gland, but neither 36/18.57 (anti-EMA-1) nor the isotype-matched control MAb 18.185 (anti-*C. parvum*) bound *B. equi* in sequential sections of infected salivary glands (Fig. 7). None of the antibodies bound the salivary glands of uninfected *Boophilus microplus* adults.

Expression of surface proteins throughout complex multiple stages of *Babesia* suggests that these conserved proteins play a fundamental role in the life cycle of the parasite within the vector tick and the mammalian host. We have conclusive evidence that the EMA-2 protein is expressed during *B. equi* stages within the midgut of nymphs and within the salivary glands of adult ticks, as well as in the erythrocyte stages in the mammalian host (13). Previous data demonstrated that EMA-2 is an orthologue of proteins in *Theileria* spp., closely related tick-transmitted protozoans (10, 13). The presence of EMA-2 expressed by *B. equi* at multiple stages within the mammalian host and the vector tick and the conservation in *Babesia* and *Theileria* parasites are consistent with a required function in the mammalian host, in the vector tick, or in both. In contrast to conclusive data for EMA-2, whether EMA-1 is expressed in the vector tick is unclear. The reactivity of MAb 36/133.97 with *B. equi* at the midgut and salivary gland stages may result from binding only to EMA-2 or may reflect coexpression of EMA-1 and EMA-2. MAb 36/18.57, with specificity solely for EMA-1, failed to bind formalin-fixed parasites even when intraerythrocytic stages, previously shown to express EMA-1 by other techniques, were examined. Development of a larger repertoire of EMA-1-specific MAbs that function in immunohistochemistry may be needed to conclusively determine whether EMA-1 is expressed by *B. equi* at different stages in ticks.

The development of morphologically distinct EMA-2-positive parasites in the nymphal midgut had a positive association with the parasitemia level of the mammalian host and the duration of tick feeding. Combined with establishing that tick feeding for at least 6 days at parasitemia levels greater than $10^{5.7}$ *B. equi* parasites per μl of blood enriches the number of *B. equi* stages in the midguts of nymphs, the use of EMA-2 as a parasite marker will also facilitate the identification of stage-specific *Babesia* proteins in the vector tick. Stage-specific *Babesia* molecules expressed in the midgut of the vector are potential targets for transmission-blocking immunity (5, 6, 16, 28). The ultimate goal of transmission-blocking immunity is to prevent infection or clinical disease by reducing tick infectivity for susceptible mammalian hosts.

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